Brain, Behavior and Evolution
Editor-in-Chief: W. Riss, New York
Publishers: S. Karger, Basel
Separatum (Printed in Switzerland)

Brain Behav. Evol. *11*: 60–72 (1975)

1975 c
187

Tests for Left-Right Chemospecificity in Frog Cutaneous Nerves[1]

Margaret Y. Scott and R. W. Sperry

Division of Biology, California Institute of Technology, Pasadena, Calif.

Key Words. Neurospecificity · Cutaneous nerves · Neuroregeneration

Abstract. Studies of the effects of competitive reinnervation by left and right cutaneous nerves were carried out in *Rana pipiens* in an effort to test for the existence of left-right biochemical differentiation of neural tissue. The dorsal cutaneous nerves were cut, transposed and allowed to regenerate under different conditions of denervation and competition and the resultant reinnervation and reflex patterns were determined by behavioral and electrophysiological mapping techniques. Contrary to earlier findings, there was no indication in the present investigation that the growth patterns were influenced by the laterality of the fibers. Other factors such as availability of terminal sites created by complete or partial denervation and the tendency of fibers to seek terminal connections seemed to override any qualifications imposed by the existence of lateral specificity on the formation of terminal contacts.

Introduction

Whether the cytochemical differentiation of the nervous system involved in regulating the developmental patterning of nerve fiber pathways and connections includes lateral chemical specification of bisymmetric structures on left and right sides has yet to be determined. The possible existence of some form of left-right biochemical specificity is suggested by the discriminative precision with which fibers of the mammalian optic nerve divide at the chiasm, and many other patterns of fi-

[1] Supported by NIH grant MH 03372, USPHS grant GM 00086, and the Evelyn Sharp Graduate Student Scholarship in Behavioral Biology. The technical assistance of Peter Jonkhoff is gratefully acknowledged.

ber decussation in central nerve tracts; the innate cerebral asymmetry in man [GESCHWIND and LEVITSKI, 1968; WITELSON and PALLIE, 1973], the lateralization of song control in avian species [NOTTEBOHM, 1971] and certain other anatomical asymmetries like that of the habenular nucleus in anura [BRAITENBERG and KEMALI, 1970; MORGAN *et al.*, 1973; SCOTT, 1973].

On the other hand, many studies on nerve development and regeneration involving left-right interchanges have failed to disclose evidence in support of such specificity [SPERRY, 1945, 1951; SPERRY and MINER, 1949; HIBBARD, 1965; GAZE, 1970; JACOBSON, 1970]. In particular, the readiness with which surgically transposed right and left nerves have been found to connect to mirror structures on the wrong side, both in the centers and periphery has seemed to point to the absence of lateral specification. Such specification is not required to account for the aforementioned anatomical asymmetries of the brain which can conceivably be explained in other terms, as can also the selective decussation at the midline [SPERRY, 1963].

In view of the foregoing, the report of FRANZISKET [1959] describing competitive cutaneous reinnervation experiments in frogs with nerves from left and right sides is of outstanding interest in that it has been taken to present a definite demonstration of lateral specificity. Ipsilateral and contralateral nerves, after being cut and surgically deflected into the same cutaneous field, were observed to display differential growth effects depending on the laterality of the competing fibers. In other instances, fiber outgrowth was found to be severely retarded when inserted into neutral tissue of the opposite side. The present study was an attempt to confirm FRANZISKET's findings. Additional related observations on the growth behavior of nerves deflected into foreign cutaneous environments are also included. In general, the present findings on *Rana pipiens* fail to confirm the earlier results of FRANZISKET [1959] on the closely related *Rana esculenta*. A brief presentation of the findings was made earlier [SCOTT, 1972].

General Procedures

Adult *R. pipiens* 7.5–10 cm in length were used throughout. The medial rami of the dorsal cutaneous nerves of this species grow out of the fascia close to the midline and lie rather loosely in the dorsal lymphatic sac (fig. 1a), a feature facilitating lateral crossing of cutaneous nerves. There was no apparent abnormal

stretch on the nerves as a result of their being deflected into contralateral cutaneous fields. ADRIAN *et al.* [1931] estimated that each of these nerve branches is comprised of about 50 sensory fibers. When describing surgical and electrophysiologcial recording procedures in the present investigation, we refer to whole nerve bundles and not to individual fibers.

Surgery was performed with the aid of a low-powered binocular dissecting-scope. Each animal was anesthetized in a glass container with ether vapor prior to surgery. After operation, the animals were housed three to four in 5-gallon terraria and maintained on a diet of live meal worms offered three times a week.

Drawings traced from enlarged photographs of each specimen showing cuticular pigmentation, lateral ridges and other distinguishing features were marked with stimulation points. Wiping reflexes were elicited by touching or local stroking of the skin with thin bristles exerting 1–4 g force. Behavioral responses were recorded two to three times a week after the onset of the first response signalling contralateral innervation of the skin and were continued until the time of electrophysiological recording.

Compound action potentials were recorded from the cutaneous nerves monopolarly with platinum electrodes during tactual stimulation. Each nerve was lifted out of the skin through a small transverse slit at the midline and mounted on the recording electrode. The reference electrode was attached to the forelimb of the animal. Signals were amplified and displayed on an oscilloscope with simultaneous audiomonitoring. During recording sessions the animals were anesthetized with ether and also paralyzed with an intramuscular injection of 50 μl of *d*-tubocurarine. The animals were reanesthetized at the first indication of arousal.

Experiment 1: Reciprocal Cross of Dorsal Cutaneous Nerves

Surgery

The dorsal cutaneous nerves of the midtrunk were exposed bilaterally through a longitudinal midline incision. The dorsomedial rami of left and right spinal nerve V were then sectioned distally where they enter the integument and the freed ends were crossed to a symmetrical region on the opposite side of the back (fig. 1b). In one group of five animals, the cut ends of the nerves were sewn to the subcutaneous fascia with surgical silk (60 μm diameter). In another group of six, the nerves were anchored with a tungsten needle in the intended position while the deflected skin was carefully replaced, after which the needle was withdrawn. A similar surgical procedure was performed in a third group of ten animals except that the freed tips of the crossed nerves in these cases were inserted intradermally with a fine tungsten needle into the inner layer containing the nerve plexus and blood vessels.

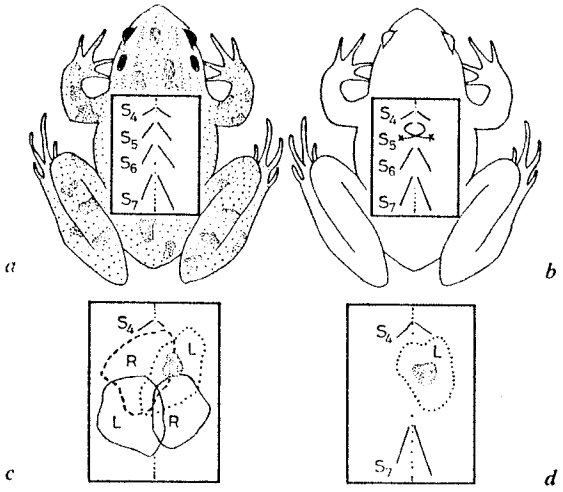

Fig. 1. a Dorsal cutaneous rami of spinal nerves (4–7) shown in cutout as they appear after emergence from dorsal musculature. *b* Reciprocal cross-attachment at S5. *c* Detail showing typical regenerated fields of crossed nerves (dashed and dotted lines) and adjacent S6 normal nerves as mapped electrically. Stippled area represents behaviorally responsive region of left (contralateral) hindlimb prior to electrical mapping. Analogous region for right nerve has been omitted for simplicity. *d* Behaviorally responsive region of crossed left nerve from same frog before (central stippled area) and after elimination of overlapping innervation.

Observations

No difference in reinnervative efficacy was noted for the two nerve-cross procedures used in the first two groups. In both, the misdirected wipe responses began to appear among the residual ipsilateral responses in the reinnervation area on about the 15th postoperative day. The area yielding crossed responses enlarged gradually to cover an average area of 0.18 cm^2 where it stabilized toward the end of the fourth week in the eight cases that survived, four from each group. In the third group of animals, the crossed responses were obtained earlier, the first ones appearing as early as 1 week after operation. Also, by the end of the fourth week, in all eight cases that survived, the region evoking crossed responses had spread over an area slightly larger than in the previous two groups averaging 0.28 cm^2. Ipsilateral responses were interspersed among the contralateral responses throughout the reinnervated regions.

The dimension of the sensory fields for the crossed and neighboring uncrossed dorsomedial rami were compared by electrical mapping in eight (four total from groups 1 and 2 and four from group 3) of the 16 survivors 5–7 weeks after surgery. The extent of the crossed fields mapped electrophysiologically were found in six of these eight frogs not to differ significantly from those of adjacent rostral and caudal ipsilateral dorsomedial rami (fig. 1c). In the remaining two cases, the regenerated cutaneous fields were approximately one half to three fourths as large as the adjacent ipsilateral nerves. Although contralateral reflexogenous zones of group 3 animals were noticeably larger than those of groups 1 and 2, reinnervated regions did not differ in size between the groups when measured electrophysiologically. Contrary to the conclusion from previous studies that fibers of opposite laterality exhibit mutual repulsion in growth, extensive overlap of cutaneous fields of indigenous and deflected contralateral nerve bundles was evident in all the present cases.

As can be seen in figure 1c, the area innervated by the transposed nerve mapped electrically was considerably larger than the same area mapped by behavioral reactions. This discrepancy can conceivably be explained on the basis that reinnervation was insufficient or somehow substandard in outer areas for effective spatial and/or temporal summation in the firing of central neurons; or that some form of central inhibition (activated, perhaps, by simultaneous stimulation of ipsilateral fibers) suppressed the latent behavioral response.

Isolation of Reinnervated Zones

The following procedure was undertaken in an effort to test some of these possible alternatives. All innervation save that from the crossed nerve was eliminated from a large section of reinnervated skin in each of the remaining eight frogs, by making an incision through the skin surrounding the area of the test nerve and transecting the adjacent ipsilateral nerves. Behaviorally responsive areas determined in this manner with overlapping ipsilateral innervation eliminated were much expanded and were comparable to those mapped electrophysiologically. Figure 1d shows reflexogenous zones mapped from the same animal just before and a day after surgical isolation of the crossed nerve. The results imply that the full extent of the reinnervated region as determined electrically was potentially functional also for behavioral responses. Apparently the intermixed ipsilateral fibers in the periphery of the reinnervated area

were responsible for preventing behavioral manifestation by some mechanism of reciprocal inhibition.

Spinal Section

The competitive reinnervative effects described by FRANZISKET [1959] were obtained in spinal animals. It seemed unlikely, but not impossible that the disparity between his results and those reported here were attributable to the effects in his animals of the spinal transection. Accordingly, the spinal cord was severed in two additional animals at the level of spinal nerve 1, between the first and second vertebrae, on the 30th day after nerve crossing. Mechanical stimulation and application of 10% AcH to the skin 0.5, 4, 24, 48 and 72 h after spinal surgery failed to elicit satisfactory localizing responses although the simpler extensor thrust was well executed. Cutaneous stimulation also failed to produce good localizing wipe responses in six normal animals after spinal section.

On the third day after spinal surgery, electrophysiological mapping of the transposed and adjacent nerves showed cutaneous receptive fields of these frogs to be essentially identical to those obtained in animals without spinal section. The condition of the spinal animals progressively deteriorated and precluded further testing and the animals were given a lethal dose of ether on the third day.

Experiment 2: Competitive Reinnervation of Denervated Skin by Nerves of Opposite Laterality

Surgery

An incision through the skin was made unilaterally in seven frogs and extended with scissors to isolate a rectangular area of skin approximately 1.1×2.0 cm covering the dorsolateral trunk between midline and lateral ridge and including the insertion points of the fourth and fifth spinal nerves. The incised skin was elevated and the two attached cutaneous nerves were transected where they enter the skin. The anterior nerve was rejoined to the center of the rostral half of the denervated patch. Instead of reconnecting the posterior nerve, the corresponding contralateral nerve trunk was freed through the same incision, sectioned distally and crossed over to the caudal half of the denervated rectangle

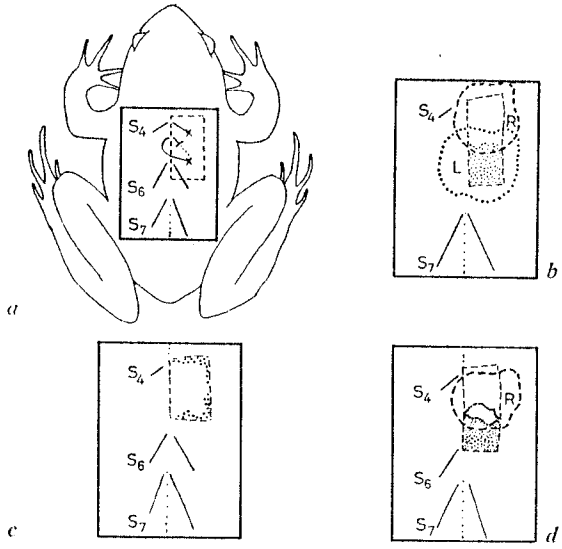

Fig. 2. a Initial operation: section and reattachment of right nerve S4 to anterior half of circumsected skin patch (dashed line), removal of right cutaneous nerve S5 and its replacement by contralateral S5. Different features of resultant reinnervation from three separate animals are shown in b, c, and d. *b* Electrophysiological maps for right (dashed line) and left (dotted line) regenerated nerves. Stippled area represents region of contralateral behavioral responses prior to electrical mapping. *c* Peripheral stippling indicates residual ipsilateral responses after section of S4 and S5. *d* Dashed line circumscribed region reinnervated by ipsilateral S4 as mapped electrically. Stippling shows reflexogenous zone of regenerated contralateral S5 prior to section of ipsilateral S4. Dotted line indicates expansion of reflexogenous zone after section of ipsilateral S4.

where the cut end was inserted intradermally near the middle of the caudal half. The incised skin patch was then carefully replaced in its original position (fig. 2a). The frogs were kept on a moist pad until the reimplant seemed secure before being returned to the home terraria. Two frogs had to be discarded because the patches failed to adhere. Of the remaining five, the reimplants healed without complication. The aim of the surgical procedure was to determine the effect on reinnervation of having a contiguous competitive cutaneous field supplied with regenerating sensory fibers of opposite laterality.

Observations

Behavioral testing was carried out daily until the onset of the first responses, then on every second-fourth day until the time of electrophysiological mapping. Sensitivity to tactile stimulation within the isolated rectangle began to return in all cases 11–12 days after surgery. Normal and misdirected responses appeared at about the same time from rostral and caudal zones respectively, and the sensitive area spread to fill the incised area by the 30th day. Contralateral reflexes remained confined to caudal portions of the patch, and normal reflexes were generally restricted to the rostral portion (fig. 2b) except for occasional points along the caudal and caudolateral incision lines that presumably resulted from ingrowth of fibers from outside the incision boundary. Contralateral and ipsilateral behavioral responses were not intermixed within the incised skin patch except for the above-mentioned peripheral regions. Contralateral responses were occasionally elicited from points caudal to the incision line in one case.

Electrophysiological mapping was carried out on two of the surviving four frogs 5 weeks after surgery. The results showed that the two test nerves had each partially invaded the territory innervated by the other to form a common overlapping field (fig. 2b). As can be seen in figure 2b, both ipsilateral and contralateral nerves were found to extend beyond the incision scars to reinnervate regions occupied by adjacent nerves. After the recording sessions were completed, both nerves were cut and behavioral mapping was repeated the next day for residual responses. The stippled area in figure 2c represents the area from which wipe reflexes were obtained – all with the ipsilateral limb. The results confirm the assumption that ipsilateral fibers from adjacent tissue had grown across the incision line. Although the terminal fields of the two regenerated nerves, one from each side of the body, overlapped extensively by electrical mapping, the behaviorally reactive zones for ipsilateral and contralateral limbs had been clearly demarcated, excepting for the above-mentioned regions immediately adjacent to the incision lines. Unlike the results reported in the first experiment, ipsilateral responses were not interspersed within the main body of contralateral responses.

The remaining two frogs were used to map the extent of the regenerated field of the anterior nerve electrophysiologically. After this was completed, the rectangular patch was reincised and the ipsilateral nerve was ablated leaving intact the surgically crossed posterior nerve as the sole innervation to the incised area. Testing of localizing responses the

next day revealed that terminal fibers of the contralateral nerve extending into the overlap territory formerly occupied also by the ipsilateral anterior nerve were sufficient to trigger wiping reflexes (fig. 2d). Figure 2d also shows the regions from which these misdirected responses were elicited from the same animal immediately prior to the electrophysiological recording session.

Experiment 3: 180° Rotation of Skin Patch

Surgery

A rectangular piece of skin 2.0×2.2 cm extending the width between the lateral ridges and encompassing the cutaneous fields of the dorsomedial rami of the fourth and fifth spinal nerves was cut free, rotated 180° and replaced leaving the two pairs of attached nerves intact.

Observations

Mechanical stimulation of the rotated patch elicited contralateral wiping reflexes on the day following rotation. The reversed reflexes were obtained consistently from the patch until the attached nerves were sectioned 1–3 months later. From the 26th day onward, normal reflexes were occasionally elicited along border regions, presumably a result of fiber ingrowth across the incision scars. Following section of the attached nerves at either 32 (two frogs) or 96 (two frogs) days after operation, appropriate ipsilateral responses were readily elicited 2–3 mm inside all borders of the rectangle. Again it was evident that ipsilateral cutaneous fibers had invaded and functionally innervated cutaneous regions of opposite laterality that already were predominantly supplied by their original contralateral fibers.

After section of all medial nerve branches supplying the rotated skin reactive areas were found to persist along the lateral regions of the patch and were similar to those found in normal animals after elimination of the same medial cutaneous nerves. These medial branches of the dorsal cutaneous rami constitute the main nerve supply of the dorsal integument. However, the dorsolateral branch, another division of the dorsal cutaneous ramus, innervating the adjacent side skin around the lateral ridge, extends medially to overlap somewhat with the dorsal medial nerves. It was evident that even fibers of the dorsolateral branches had reinnervated the foreign contralateral patch with what appeared to be normal terminal connections.

There was no indication that regenerating neurites of either dorsomedial or dorsolateral branches of the dorsal cutaneous rami were inhibited in their growth as they advanced across the incision into contralateral skin to make functional connections in areas already innervated by neural tissue of the opposite side.

Experiment 4: Unilateral Denervation of the Dorsal Trunk

It is conceivable that nerve rami originating on one side of the midline restrict their innervation fields mainly to the same side because of some growth limitations, e.g., a maximum length particular fibers can attain. To test this hypothesis, a major portion of the dorsum was unilaterally denervated by sectioning the innervating dorsocutaneous nerves to see if contralateral fibers would be stimulated to cross the midline and extend abnormal distances into the opposite side.

Surgery

A longitudinal incision about three dermatomes long was made along the right lateral ridge in eight frogs. Transverse cuts to the midline at both ends of the rostrocaudal incision produced a rectangular skin flap which was folded back to expose the medial dorsal cutaneous nerves. All nerves connecting with the flap as far distally and proximally as possible were sectioned and removed after which the denervated flap was replaced.

Observations

Behavioral mapping carried out several hours after operation showed that the reflexogenous fields of the left dorsal cutaneous rami extended slightly beyond the midline and were similar to those previously determined electrophysiologically in normal animals. Between 21 and 25 days after unilateral denervation it was possible to elicit contralaterally misdirected cutaneous localizing reflexes with the left limb from most of the previously denervated flap on the right side. The exception was a 2-mm strip along the lateral ridge where ipsilateral responses predominated and some remaining insensitive loci were occasionally encountered. Recutting around the flap and redividing any nerves from the right side which were found to have regenerated eliminated all ipsilateral respon-

ses. Unresponsive regions were again observed along the lateral margin after this second operation.

After another period of 3–4 weeks, electrophysiological recording from the left midline cutaneous nerves indicated that two of every three nerves from which recordings were made had expanded their terminal fields across the midline all the way to the opposite lateral ridge to cover the entire dorsum. Behaviorally unresponsive zones remained along the lateral ridge even though electrophysiology showed that some of these regions were indeed innervated.

Discussion

No evidence was obtained in the foregoing that would demonstrate the existence of lateral cytochemical specificity. In all cases regenerating fibers of left and right cutaneous nerves appeared to grow with equal readiness into overlapping fields of neighboring nerves regardless of fiber laterality. The differential inhibition of growth exerted by fibers of one laterality on fibers from the opposite side as described earlier by FRANZISKET [1959] under very similar experimental conditions was not found in this investigation.

The disparity between FRANZISKET's results and those obtained here remains to be explained. As far as can be ascertained, surgical and behavioral testing methods employed in the two studies were similar. FRANZISKET, however, used chronic spinal animals while the spinal animals used in the present investigation were acute preparations. Perhaps this factor was critical though it seems unlikely as does the possibility that such closely related species as *R. esculenta* and *R. pipiens* would have developed such a difference for the patterning of peripheral connections.

The appropriate pattern of cutaneous innervation at the midline can conceivably be accomplished during development without reciprocal lateral repression of fiber outgrowth and in fact may argue against such repression. In normal frogs there is considerable right-left overlap of cutaneous fibers at the midline although this midline overlap is by no means of the magnitude found between adjacent dermatomes. One might ask how cutaneous fibers normally are restricted from growing more extensively across the midline since Experiment 4 has shown that fibers from one side do have potential for innervating the entire dorsum. The an-

swer remains to be determined but may conceivably be found in the interplay of such factors as timing of fiber outgrowth, the tendency of growing fibers to fill nearest areas first, limitation on number of active terminals per fiber, maximum density of terminal sites per unit area, and the like, without need to invoke lateral chemospecificity.

The present results are consistent with numerous surgical rearrangement experiments that have indicated an equal interchangeability of neural structures from one side to the other under many conditions. None of these experiments, however, including the present, firmly exclude the possible existence of a left-right specificity that may yet be revealed by different kinds or more refined tests. Recent findings on neonatal rats and hamsters by LUND and LUND [1973] and SCHNEIDER [1973] during the course of the present investigations seem suggestive but not definitive and one can only conclude at this point that the question still remains open.

Since left and right cutaneous nerves normally overlap somewhat across the midline the observed absence of misdirected reflexes along the midline and their presence after ipsilateral denervations requires some explanation. The function of those fibers that have crossed the midline must be suppressed or inhibited in some way, presumably through concomitant stimulation of overlapping ipsilateral fibers. Differential density of fiber terminals from the two sides in conjunction with central reciprocal inhibition of the weaker of the two conflicting response tendencies offers a plausible basis by which the spinal centers determine ipsilateral vs. contralateral responses.

References

ADRIAN, E. D.; CATTELL, M., and HOAGLAND, H.: Sensory discharges in single cutaneous nerve fibres. J. Physiol., Lond. *72:* 377–391 (1931).

BRAITENBERG, V. and KEMALI, M.: Exceptions to bilateral symmetry in the epithalamus of lower vertebrates. J. comp. Neurol. *138:* 137–146 (1970).

FRANZISKET, L.: Verhandlungen der Deutschen Zoologischen Gesellschaft Münster/Westf., 1959, pp. 139–145.

GAZE, R. M.: The formation of nerve connections (Academic Press, London 1970).

GESCHWIND, N. and LEVITSKI, W.: Human brain: left-right asymmetries in temporal speech regions. Science *161:* 186–187 (1968).

HIBBARD, E.: Orientation and directed growth of Mauthner's cell axons from duplicated vestibular nerve roots. Expl. Neurol. *13:* 289–301 (1965).

JACOBSON, M.: Developmental neurobiology (Holt, Rinehart & Winston, New York 1970).

LUND, R. D. and LUND, J. S.: Reorganization of the retinotectal pathway in rats after neonatal retinal lesions. Expl. Neurol. *40:* 377–390 (1973).

MORGAN, M. J.; O'DONNELL, J. M., and OLIVER, R. F.: Development of left-right asymmetry in the habenular nuclei of *Rana temporaria*. J. comp. Neurol. *149:* 203–214 (1973).

NOTTEBOHM, F.: Neural lateralization of vocal control in a passerine bird. I. Song. J. exp. Zool. *177:* 229–262 (1971).

SCHNEIDER, G. E.: Early lesions of superior colliculus: factors affecting the formation of abnormal retinal projections. Brain Behav. Evol. *8:* 73–109 (1973).

SCOTT, M. Y.: On the problem of left-right specificity of neural tissue. Calif. Inst. Technol. Div. Biol. Ann. Rep. *81:* 100 (1972).

SCOTT, M. Y.: Anatomical asymmetry in frog brain. Calif. Inst. Technol. Div. Biol. Ann. Rep. *82:* 56 (1973).

SPERRY, R. W.: Restoration of vision after crossing of optic nerves and after contralateral transplantation of eye. J. Neurophysiol. *8:* 15–28 (1945).

SPERRY, R. W.: Mechanisms of neural maturation; in STEVENS Handbook of experimental psychology, pp. 236–280 (Wiley, New York 1951).

SPERRY, R. W.: Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc. nat. Acad. Sci. USA *50:* 703–710 (1963).

SPERRY, R. W. and MINER, N.: The formation within sensory nucleus. V. of synaptic associations mediating cutaneous localization. J. comp. Neurol. *90:* 403–424 (1949).

WITELSON, S. F. and PALLIE, W.: Left hemisphere specialization for language in the newborn. Neuroanatomical evidence of asymmetry. Brain *96:* 641–646 (1973).

MARGARET Y. SCOTT, Division of Biology, California Institute of Technology, *Pasadena, CA 91125* (USA)